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ANTIBIOTICS IN AGROECOSYSTEMS: STATE OF THE SCIENCE

Antibiotics and Antibiotic Resistance in Agroecosystems: State of the Science

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Abstract

We propose a simple causal model depicting relationships involved in dissemination of antibiotics and antibiotic resistance in agroecosystems and potential effects on human health, functioning of natural ecosystems, and agricultural productivity. Available evidence for each causal link is briefly summarized, and key knowledge gaps are highlighted. A lack of quantitative estimates of human exposure to environmental bacteria, in general, and antibiotic-resistant bacteria, specifically, is a significant data gap hindering the assessment of effects on human health. The contribution of horizontal gene transfer to resistance in the environment and conditions that might foster the horizontal transfer of antibiotic resistance genes into human pathogens also need further research. Existing research has focused heavily on human health effects, with relatively little known about the effects of antibiotics and antibiotic resistance on natural and agricultural ecosystems. The proposed causal model is used to elucidate gaps in knowledge that must be addressed by the research community and may provide a useful starting point for the design and analysis of future research efforts.

Core Ideas

- We propose a simple causal model of antibiotics and antibiotic resistance in agroecosystems.
- Lack of data on human exposure to resistance in the environment hinders risk assessment.
- The contribution of horizontal gene transfer to resistance in the environment requires research.
- Antibiotics in environment may mediate effect of antibiotic use on resistance in the environment.
- Effects of antibiotics and antibiotic resistance on natural and agricultural ecosystems are largely unknown.

Antibiotic resistance is now considered by authorities in the United States and by international experts to be one of the paramount public health challenges of our time (Centers for Disease Control and Prevention, 2013; World Health Organization, 2014). In 2014, a committee of the US executive branch issued a sobering report urging rapid action to address the impending antibiotic resistance crisis (President's Council of Advisors on Science and Technology, 2014). The role of food, community, and healthcare exposure in the transmission of antibiotic-resistant pathogens has generated significant discussion and research effort (White et al., 2002; Chastre, 2008; Walsh and Fanning, 2008; Koluman and Dikici, 2013; Williamson and Heffernan, 2014). Due to the widespread use of antibiotics in agriculture, there has also been increasing controversy, concern, and related research on agricultural contributions to dissemination of antibiotics and antibiotic resistance. The natural environment could play an important role as a reservoir and a source of resistance (Martinez, 2009; Berendonk et al., 2015), particularly where it is affected by agriculture (You and Silbergeld, 2014).

The use of antibiotics is intrinsically linked to the occurrence of bacterial resistance against these compounds. Antibiotics are widely used for the treatment and prevention of diseases in humans, animals, and less commonly in crop plants. Many antibiotics are also used to promote growth and enhance feed efficiency in animal agriculture. After use, antibiotic residues as well as antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are introduced to soil and

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Abbreviations: ADI, acceptable daily intake; ARB, antibiotic-resistant bacteria; ARG, antibiotic resistance gene; HGT, horizontal gene transfer; MGE, mobile genetic element; MLS_B, macrolide, lincosamide, streptogramin B; MRSA, methicillin-resistant *Staphylococcus aureus*

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water through direct excretion, land application of biosolids or animal manures as fertilizers, and irrigation with wastewater or treated effluents. The presence of active antibiotic compounds in environmental compartments may also select for resistance, either intrinsic or acquired through transferable elements, in native environmental bacteria. Human populations and biota in natural and agricultural ecosystems may then be exposed to these increased levels of ARB. The pathways of exposure are complex and poorly understood, and the effects of these exposures on human health, ecosystem function, and productivity of agricultural systems remain unclear.

In this paper, we explore the evidence that antibiotic use affects antibiotics and ARB or ARGs in agroecosystems, with negative consequences for humans, natural ecosystems, and agricultural productivity. Our intent is not to provide a comprehensive review of all available research but rather to highlight relevant findings, identify major knowledge gaps, and put forward priorities for future research. To do this, we rely on a causal modeling approach, known as directed acyclic graphs, which can be used to visualize ordered causal relationships between factors in complex systems. These models are finding increasing use in diverse fields, such as epidemiology and psychology, to minimize bias and make assumptions explicit when estimating causal effects from data (Shrier and Platt, 2008; Joffe et al., 2012).

We propose a causal model depicting the relationships involved in environmental dissemination of antibiotics and antibiotic resistance and related causal effects (Fig. 1). This model shows the following hypothesized relationships: intentional use of antibiotics results in increased levels of antibiotics (arrow 1), ARB (arrow 2), and ARGs (arrow 3) in agroecosystems. Residues of antibiotics cause an enrichment of ARB in the environment (arrow 4). Although environmental ARB and ARGs are distinct, they are intrinsically linked with unknown or mixed causal direction (arrow 5). We consider the effects of both antibiotics and ARB in agroecosystems on three important outcomes: human health (arrows 6 and 9, respectively), ecosystem function (arrows 7 and 10, respectively), and agricultural system productivity (arrows 8 and 11, respectively). This type of formal graphical assessment may aid in building consensus within the research community around which studies are the most urgently needed in the future and the most appropriate design and objectives of such studies. In the following sections, we use this causal model as a structure for discussion of current understanding and to identify major knowledge gaps for each causal step.

Effect of Antibiotic Use on Antibiotics in Agroecosystems (Arrow 1)

Estimates of agricultural animal and human antibiotic use from Canada, Denmark, and Australia (Bager, 2000; Canadian Integrated Program for Antimicrobial Resistance Surveillance, 2008; Joint Expert Technical Advisory Committee on Antibiotic Resistance, 2009) indicate that antibiotics administered to animals account for a majority of all antibiotics used by weight. The potential for release of antibiotics into agroecosystems is therefore large. It is then not surprising that over the past 15 yr a large number of antibiotics from a wide range of classes, including macrolides (erythromycin, tilmicosin, tylosin, tilmicosin), lincosamides (lincomycin), sulfonamides (sulfadiazine, sulfamethazine, sulfamethoxazole), thiamphenicol analogs (chloramphenicol, florfenicol), fluoroquinolones (ciprofloxacin, enrofloxacin, norfloxacin), tetracyclines (chlortetracycline, doxycycline, oxytetracycline, tetracycline), and other classes (monensin, trimethoprim) have been detected in agroecosystems (Campagnolo et al., 2002; Hamscher et al., 2002; Kolpin et al., 2002; Halling-Sorensen et al., 2003; Aga et al., 2005; Batt et al., 2006; McKinney et al., 2010; Watanabe et al., 2010; Bartelt-Hunt et al., 2011; Pruden et al., 2012; Zhang et al., 2013). These antibiotics have been detected in agroecosystems across the world, including North and South America, Europe, and Asia, and in a variety of environmental compartments, including soils, surface waters, sediments, and biota such as plants and earthworms.

Although large quantities of data have been generated on the fate and occurrence of veterinary-use antibiotics in agroecosystems, our understanding of their overall spatial and temporal distribution is still quite limited. This research has often been performed in small geographical areas and was frequently restricted to analysis of nonsystematic grab samples. Furthermore, little monitoring has occurred for many individual veterinary-use antibiotics that are less widely used but also have the potential to enter agroecosystems. Moreover, limited information is available on the fate and occurrence of human-use antibiotics that will enter the environment when biosolids are applied to agricultural soils as a fertilizer. The use of exposure prediction models provides a tool to understand exposure over space and time and to evaluate the likely occurrence of antibiotics that have yet to be monitored (Schowanek and Webb, 2002; Wajsman and Ruden, 2006; Boxall et al., 2014; Iatrou et al., 2014). Such modeling efforts can consider the quantities of antibiotics used, the rate of metabolism before release, the mechanism and temporal

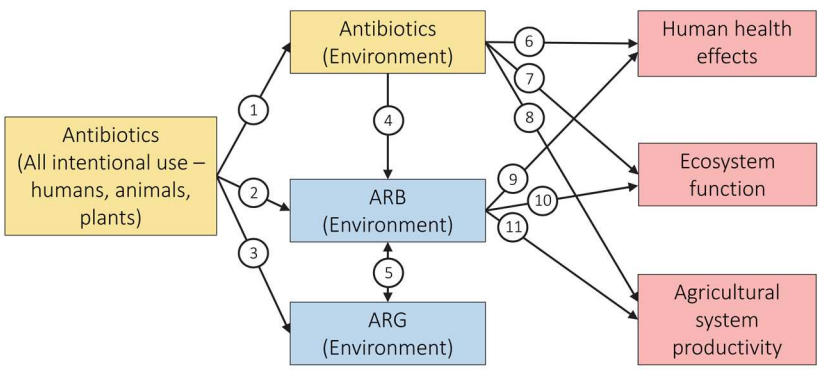

Fig. 1. Conceptual causal model depicting hypothesized effects of antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in agroecosystems and the environment. Intentional use of antibiotics results in increased levels of antibiotics (1), ARB (2), and ARGs (3) in agroecosystems. Residues of antibiotics cause an enrichment of ARB in the environment (4). ARB and ARGs, though distinct, are intrinsically linked with unknown or mixed causal direction (5). The effects of both antibiotics and ARB in agroecosystems on human health (6 and 9), ecosystem function (7 and 10), and agricultural system productivity (8 and 11) are shown. Yellow represents active antibiotic compounds. Blue represents resistance elements. Pink represents the outcomes of interest.

variations of release into the environment, and parameters governing the movement and persistence of the compound and its metabolites once in the environment. Figure 2 presents the results of a simple modeling exercise, performed for this paper, attempting to evaluate the potential level of exposure of agricultural soils to current-use veterinary antibiotics. Concentrations of the antibiotics in soils were calculated using algorithms proposed by the Committee for Medicinal Products for Veterinary Use (CVMP, 2007). These algorithms use information on typical treatment doses and durations for products containing the antibiotic, average masses of the treated animal, animal housing characteristics, manure/slurry characteristics, and limits on fertilizer application to soils. Because limited information is available in the public domain on the half-lives of antibiotics in manures and slurries and on the activity of transformation products, a total residue approach was used that assumes no degradation of the parent compound. Estimated concentrations are therefore likely to be an upper limit for some antibiotics. The results of the model indicate that no monitoring data currently exist for many of the compounds where exposure is predicted to be high.

Effect of Antibiotic Use on Environmental Antibiotic Resistance (Arrows 1–4)

The use of antibiotics in animal production, followed by land application of manure, is common practice in the developed world and provides a direct route for introduction of antibiotics, ARB, and ARGs into agroecosystems. Conventional animal agriculture is associated with an increase in ARB and/or ARGs in manure lagoons, as compared with organic production practices (e.g., Jindal et al., 2006; Peak et al., 2007). In manure-amended soil, multiple studies indicate a transient increase in ARB and ARGs (Jensen et al., 2002; Sengelov et al., 2003; Zhou et al., 2010). Similarly, investigations of tetracycline and sulfonamide resistance genes in a river in Colorado found that sites closer to dairy farms had higher concentrations of ARGs (e.g., Pruden et al., 2006). Overall, it is clear that animal production activities, particularly those that permit antibiotic use, can increase the abundance of ARB and ARGs in agroecosystems.

In aquaculture, antibiotics are most often delivered as a supplement in fish food but are also added to the water or occasionally delivered via injection (Cabello, 2006; Tusevljak et al., 2013). The impact of this antibiotic use on the prevalence of ARB and ARGs in aquaculture production settings has been reviewed elsewhere for salmon farming (Miranda, 2011) and for aquaculture more generally (Cabello et al., 2013). Overall, there is evidence that aquaculture activities are associated with an increase in ARB and ARGs in cultured fish and in the sediment below farms, but data to support a similar effect in surrounding environments are inconsistent (Schmidt et al., 2000; Chelossi et al., 2003; Petersen and Dalsgaard, 2003; Gordon et al., 2007; Miranda, 2011; Buschmann et al., 2012; Cabello et al., 2013; Muziasari et al., 2014; Shah et al., 2014).

A potential cause of ARB and ARGs in the environment that is often not considered in assessment of agricultural antibiotic use is application to tree crops. Streptomycin and, to a lesser extent, oxytetracycline are the primary antibiotics used on crops and trees in the United States and Europe (McManus et al., 2002). Gentamicin, kasugamycin, and oxytetracycline are used on crops and trees in Mexico and Latin America, and oxolinic acid is used in Israel. Antibiotic-resistant bacteria can be found in plant agricultural settings (Tolba et al., 2002), but observational and controlled field investigations generally agree that spraying of streptomycin on crops does not measurably enrich for streptomycin-resistant bacteria or streptomycin resistance genes on plants or in the surrounding soil (reviewed in McManus [2014]). However, streptomycin- and oxytetracycline-resistant strains of the plant pathogen *Erwinia amylovora* have been documented (Stockwell and Duffy, 2012), suggesting that the efficacy of these antibiotics for control of plant disease may be limited in the future.

Application of sewage sludge or biosolids as fertilizer is another route by which ARB and ARGs can be introduced to agroecosystems. Biosolids have been shown to contain antibiotics, ARB, and ARGs (Auerbach et al., 2007; Munir et al., 2011;

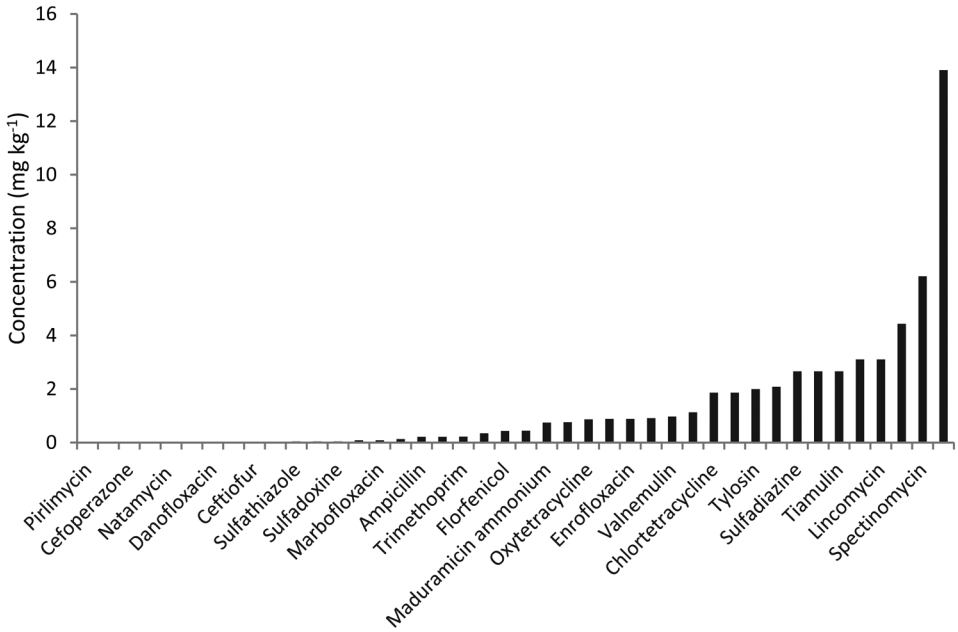

Fig. 2. Predicted concentrations of veterinary antibiotics in use in Europe in agricultural soils, calculated for this paper using a simple exposure model (CVMP, 2007), worst case treatment scenarios, and data on typical livestock characteristics and agricultural practices.

Munir and Xagoraraki, 2011), but studies examining the impact of land application of biosolids on antibiotic resistance in soil are few in number and do not provide consistent results. One study found no effect of biosolids on ARB (Brooks et al., 2007), whereas another yielded contradictory results at different field sites (Munir and Xagoraraki, 2011). Laboratory batch tests suggest that ARGs from biosolids will have half-lives ranging from 2 wk to 3 mo in soil (Burch et al., 2014), suggesting that long-term field studies are necessary to truly discern any soil effects from biosolids application. Although further study is warranted, there is no conclusive evidence that land application of biosolids results in a persistent increase in the prevalence of ARB or ARGs.

Irrigation with wastewater effluent poses another potential route for entry of antibiotics, ARB, and ARGs into agroecosystems. As with the biosolids, treated wastewater effluents are known to contain ARB and ARGs (Auerbach et al., 2007; LaPara et al., 2011; Munir et al., 2011). However, there is no evidence that this practice increases the prevalence of ARB or ARGs in environmental media, and studies specifically examining this question at sites with long-term exposure to treated effluent (years to decades) have found no effect (Negreanu et al., 2012; McLain and Williams, 2014). The use of untreated wastewater for irrigation is associated with an increase in the abundance of both microbial biomass and ARGs, but no concomitant increase in ARB was detected in a small number of studies (Dalkmann et al., 2012; Chen et al., 2014). Although the potential for dissemination of ARGs through irrigation with treated wastewater remains, the available evidence suggests that the impact of this practice on the prevalence of ARB and ARGs in the soil is minimal.

Recent mathematical models have been used to evaluate the combined influence of numerous anthropogenic sources on observed levels of ARGs found in river systems (Pruden et al., 2012; Amos et al., 2015). Amos et al. (2015) constructed a multilevel model to predict the prevalence of the Class 1 integron gene, *int1*, in the Thames River basin as a function of wastewater treatment plant outputs, land cover type, and climatic variables. The model had fairly good predictive capability at another site in the United Kingdom. Predictive models of ARB distribution have not yet been created. As with environmental antibiotics, further developments in predictive modeling of environmental resistance will improve understanding of where ARB and ARGs can be expected, thereby aiding risk assessment efforts.

Direct versus Indirect Effects of Antibiotic Use on Environmental Antibiotic-Resistant Bacteria

Antibiotic use in humans and animals is thought to increase the prevalence of antibiotic resistance in agroecosystems and environmental media through the introduction of antibiotics and subsequent selection of resistant bacterial subtypes present there (Fig. 1, arrows 1 and 4) and/or through the introduction of ARB or ARGs from anthropogenic effluent into these media (Fig. 1, arrow 2 or 3). In all likelihood, a combination of these are at play. For accurate risk assessment and mitigation efforts, it is important to understand the relative impact of these two potential pathways on the observed levels of ARB and ARGs in agroecosystems. Unfortunately, few data are available to disentangle these effects, and the evidence that is available does not support a clear conclusion.

Microcosm studies with manure-amended soil suggest that the observed effects of antibiotic use on ARB and ARGs in environmental media may be predominantly due to environmental antibiotics rather than the direct addition of these elements. For example, when manure containing sulfadiazine was added to soil, ARB retrieved did not correspond to those present in the original manure but were likely enriched from the soil (Heuer and Smalla, 2007). The addition of sulfadiazine-spiked manure also resulted in a much larger increase in the relative abundance of ARGs in manure-amended soil than did addition of antibiotic-free manure (Heuer et al., 2011). One caveat associated with these experiments was that the manure used for spiking was from untreated animals, and therefore its community composition may not be representative of the ARB present in manure from treated animals. In contrast, another study found that the addition of ARB-spiked manure resulted in percentages of resistance to macrolide, lincosamide, and streptogramin B (MLS_B) over time that were more similar to those resulting from the addition of manure from treated animals than the profiles from the addition of antibiotic-spiked manure (Zhou et al., 2010). In this case, the ARB were *Clostridium* spp. isolated from untreated manure and known to represent a substantial fraction of the MLS_B–resistant microorganisms in both types of manure. Although the studies mentioned above suggest the possibility that manure containing antibiotics or ARB may induce increases in ARB in the natural soil flora, there is some evidence that manure from untreated animals also increases antibiotic resistance in soil (Jechalke et al., 2013; Udikovic-Kolic et al., 2014).

Overall, evidence for the relative contributions of antibiotics versus ARB or ARGs released into the environment on the observed resistance levels in environmental media is limited, thus identifying an important priority for further study. There are a number of complexities likely to influence the strength of these causal relationships in a system at a given time, including source-dependent temporal variation in the release of contaminants into the environment (Daughton, 2003; Lissemore et al., 2006; Bernot et al., 2013) and differences in the stability, accumulation, and ultimate concentration of various antibiotic classes in environmental media (Aga et al., 2016). The time scale over which relationships between antibiotics and resistance in environmental media may manifest, and any nonlinearity (e.g., threshold effects) in these relationships will also affect the ability to detect them. These complexities must be carefully considered in studies attempting to assess the relationships between antibiotic use, antibiotics, and resistance in agroecosystems.

Given appropriately defined time scales, causal modeling techniques may offer resources for determining the relative contributions of the direct versus indirect effects of antibiotic use on ARB and ARGs in environmental media, particularly when experimental control of all relevant factors is not possible. Causal mediation analysis is a tool that can be used to estimate the relative contribution of mediated and direct effects of an exposure on an outcome (Richiardi et al., 2013; Pearl, 2014) and thus can be used to determine the extent to which antibiotics in the environment may mediate the effect of antibiotic use on environmental ARB or ARGs.

Relationship of Environmental Antibiotic-Resistant Bacteria and Antibiotic Resistance Genes

Distinguishing the relative contributions of ARB and ARGs to dissemination of resistance (Fig. 1, arrow 5) is crucial for risk assessment and identification of appropriate control measures and is a challenging task because ARB and ARGs are intimately connected (Fig. 3). Antibiotic-resistant bacteria contain intracellular ARGs, which in many cases can be transferred to other bacteria through horizontal gene transfer (HGT, also called lateral gene transfer), resulting in new ARB. Horizontal gene transfer can occur through cell-to-cell contact (conjugation) or via viruses (transduction), as represented by path C in Fig. 3. Intracellular ARGs can also be released, either actively or on cell lysis (Fig. 3, path A), and then incorporated and expressed within new bacterial cells (natural transformation; Fig. 3, path B), again resulting in the generation of new ARGs. In all cases, the new ARB may be the same or a completely different bacterial species from the original ARB, which may differ in, for example, environmental persistence or pathogenicity. Data attempting to resolve the relative contributions of ARB and ARGs to development and persistence of overall resistance have not been reported, but this has recently been recognized as a significant knowledge gap in peer-reviewed literature (e.g., Ashbolt et al., 2013).

Despite the intrinsic connection between ARB and ARGs, these two contaminants present different risks in environmental systems. As illustrated in Fig. 1, ARB impart direct risk to human health, whereas the risk from ARGs is indirect. Because the fate, transport, and inactivation of pathogenic bacteria have been widely studied, there is a great deal of information available about the probable fate of ARB in soil and water. Far less is understood about the transport and fate of extracellular ARGs. The persistence of extracellular DNA is well established, with reported half-lives ranging from months to years in soil environments (reviewed in Levy-Booth et al. [2007], Nielsen et al. [2007], and Pietramellara et al. [2009]). Because extracellular DNA persists through adsorption to soil or sediment particles, transport of extracellular ARGs is likely to be controlled by the movement of those particles, but to our knowledge this has not been studied directly. Adsorbed DNA is available for uptake and expression by a variety of naturally competent bacteria (Lorenz and Wackernagel, 1994).

Although ARGs have often been quantified in agroecosystems, uncertainty about how effective commonly used molecular methods are at detecting extracellular DNA (Luby et al., 2016) and whether or not they accurately reflect its potential biological activity complicates interpretation of these results. The effects of some drinking water disinfection processes on transforming activity of ARGs have been tested (Dodd, 2012), illustrating that it is possible to address this knowledge gap. Studies that address recovery efficiency for extracellular DNA adsorbed to environmental matrices and, further, that clarify the relationship between capacity for polymerase chain reaction amplification and transformation/expression efficiency could allow more accurate interpretation of existing and future data. Important considerations related to the benefits and drawbacks of various detection methods for ARB and ARGs in environmental media are reviewed in this issue (Luby et al., 2016; McLain et al., 2016).

The dissemination and the impacts of ARGs are inextricably linked to HGT; without mechanisms of transfer, ARGs and

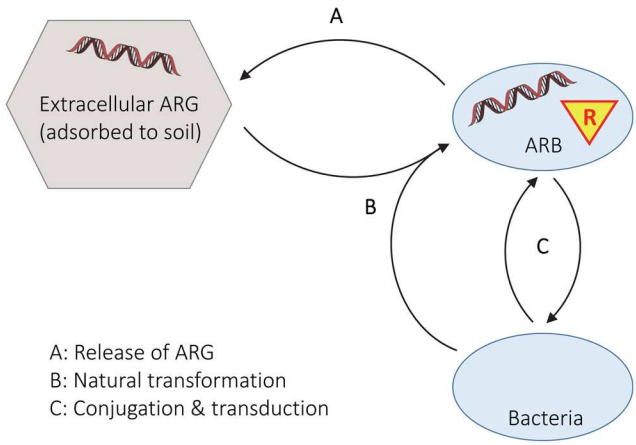

Fig. 3. Schematic representation of relationships between susceptible bacteria, antibiotic-resistant bacteria (ARB), and extracellular antibiotic resistance genes (ARGs). R (in triangle) represents resistance phenotype.

ARB would be indistinguishable in terms of their fate, transport, and impact. There is strong evidence that HGT can occur in the environment by conjugation, transduction, and natural transformation, and genome sequence analysis confirms that HGT is widespread in microorganisms (Ochman et al., 2000). Antibiotic resistance genes are found in many different types of mobile genetic elements (MGEs), whereas pre–antibiotic-era isolates tend not to have ARGs located on MGEs (Stokes and Gillings, 2011). This supports a strong effect of anthropogenic antibiotic use as well as an increased role of MGEs in dissemination of resistance. The potential effects of this are sobering because some MGEs have co-opted bacterial stress responses into regulatory processes (Beaber et al., 2004; Guerin et al., 2009). In effect, for antibiotics that trigger a stress response, the presence of that antibiotic can increase rates of gene transfer between related or unrelated cells. The generality of this phenomenon across different antibiotics, MGEs, and bacterial species is not known.

Direct evidence for the impact of HGT on the prevalence of antibiotic resistance in agroecosystems, or in any environment, has not yet been reported. Such evidence would be challenging to obtain given the complexity of HGT processes. For example, methods for blocking all mechanisms of HGT in a microbial community are not available, reducing the possibility of experimentally separating processes to study them. Furthermore, during the HGT processes, ARGs are replicated, removing any possibility of labeling or chemical tracking. Finally, the possibility of source tracking HGT does not yet exist because ARG sequences do not provide sufficient diversity to unambiguously determine origin. Each of these challenges must be addressed to accurately answer critical questions about the behavior of ARGs in environmental systems because even rare HGT events could have major impacts on the abundance, diversity, and persistence of ARB in the environment.

Potential Effects of Environmental Antibiotics (Arrows 6–8)

Effects on Human Health

We are not aware of evidence documenting human health consequences of human exposure to antibiotic residues in the

environment (Fig. 1, arrow 6), and limited information is available on the amounts of antibiotics to which humans may be exposed via the environment. However, human exposure to antibiotics from the environment is possible via a number of pathways, including ingesting contaminated food and water and breathing in contaminated dust particles. Measurable levels of antibiotic uptake from growth media into crops have been shown for a variety of plant types, including carrots (Boxall et al., 2006), cucumber (Shenker et al., 2011), lettuce (Sallach et al., 2015), and rice (Boonsaner and Hawker, 2012). A variety of agricultural-use antibiotics have been detected in drinking water, including macrolides, quinolones, and sulfonamides (Ye et al., 2007; Benotti et al., 2009; Yiruhan et al., 2010). Direct exposure of antibiotics through animal-based food products has been studied in beef (Carretero et al., 2008), eggs (Donoghue et al., 1996), honey (Sheridan et al., 2008), milk (Mitchell et al., 1998), pork (Berends et al., 2001), and poultry (Vandenberge et al., 2012). Maximum residue levels of antibiotic compounds in animal food products are largely regulated in developed countries (Passantino and Russo, 2008). Antibiotics, including sulfonamides and tetracyclines, have been detected in dust originating from livestock facilities, and it is postulated that this material could be transported very long distances (Hamscher et al., 2003; McEachran et al., 2015).

In developed countries, the documented and predicted concentrations of antibiotics in dust, soil, or water are generally low. At such levels, the degree of human exposure to antibiotics via these pathways is likely to be orders of magnitude lower than acceptable daily intake (ADI) values (Holmes et al., 2007), suggesting that direct toxic effects to humans are unlikely. In developing countries, where regulatory controls on antibiotic use are less stringent, concentrations in environmental media may be higher (Kookana et al., 2014).

Despite the increasing adoption of regulations in developing countries, the potential for antibiotic exposure via the environment may be of clinical concern. Even at low levels, environmental exposure to antibiotics could result in direct human health effects where (i) the antibiotic is also being consumed via other routes (e.g., in meat) at a level close to the ADI, (ii) the ADI for an antibiotic is very low, or (iii) in instances of antibiotic sensitivities or allergies. Furthermore, potential health effects of chronic low-dose exposures have not been investigated in humans. For example, it has been suggested that ingestion of low-level antibiotics could favor the proliferation of ARB and/or ARGs in the human microbiome. Laboratory and agricultural administration of subtherapeutic levels (i.e., doses lower than recommended levels) of antibiotics has been shown to alter the composition of the microbiome of mammalian feces and, in some cases, to select for specific ARGs (Kim et al., 2012; Looft et al., 2012; Lin et al., 2014). However, enrichment of ARGs may not always be a feature of these changes (Brooks et al., 2009; Danzeisen et al., 2011), and there is currently no evidence that similar effects occur in humans. Whether such effects are likely, particularly for antibiotic exposure from environmental sources, depends on acute or chronic exposure doses, information that is currently not known.

Effects on Ecosystem Function

Because microorganisms are the target of antibiotic action, most concerns about the effect of environmental antibiotics on ecosystem function (Fig. 1, arrow 7) have focused on the soil microbial community. A wide range of effects on soil microorganisms have been reported for many antibiotics, including β-lactams, fluoroquinolones, sulfonamides, and tetracyclines, at or close to environmentally relevant concentrations. Microcosm studies of soil systems indicate that antibiotic exposure can induce changes in microbial biomass, community structure (characterized by phospholipid fatty acid analysis, 454 sequencing, or polymerase chain reaction–denaturing gradient gel electrophoresis), and effects on functional endpoints, such as substrate-induced respiration, iron reduction, N-mineralization, nitrification, and potential to degrade other anthropogenic substances (Schmitt et al., 2004; Hammesfahr et al., 2008; Kotzerke et al., 2008; Gutiérrez et al., 2010; Kleineidam et al., 2010; Monteiro and Boxall, 2010; Kotzerke et al., 2011; Solis et al., 2011; Toth et al., 2011)

Antibiotics may also have off-target effects on other species and other environments. Aquatic algal and cyanobacterial species are known to be extremely sensitive to many antibiotics, with impacts on cell growth and yields occurring at or close to environmentally relevant concentrations (Guo et al., 2015). Although antibiotics can also affect invertebrates and fish, these effects are generally seen at concentrations many orders of magnitude greater than occur in the environment (Wollenberger et al., 2000; Isidori et al., 2005). Data also indicate that wildlife such as birds and bats can accumulate nonantibiotic pharmaceutical compounds through the food chain and can be affected (Bean et al., 2014); however, the importance of accumulation of antibiotics in tissues of wildlife species is not known.

Because these compounds rarely occur as single compounds in the environment, the study of mixture toxicity is very important yet is understudied. In limited research, mixture effects have been shown to be synergistic, antagonistic, or additive depending on the compounds mixed (Yang et al., 2008; Liu et al., 2014). This may result in increased ecosystem risk at environmentally relevant concentrations (Gonzalez-Pleiter et al., 2013). Not only do mixtures occur with multiple antibiotics, they also occur with other environmental toxins, including metals and their metabolites. Studies suggest that these interactions also affect mixture toxicity (Majewsky et al., 2014; Yu et al., 2015). For additional discussion of this important issue, see Aga et al. (2016) in this issue.

Effects on Agricultural Systems

In addition to the potential impacts of antibiotics on human health and natural ecosystems, impacts on agricultural systems (Fig. 1, arrow 8) must be considered. The beneficial consequences of antibiotics in agroecosystems, such as growth promotion and disease prevention and treatment, are well known and should be considered in an overall evaluation of agricultural practices; however, we focus here on possible unintended consequences of antibiotic residues in agroecosystems on agricultural productivity.

Many soil microbial functions are essential for efficient plant growth. For example, the rate of soil organic material decomposition by soil microorganisms governs the supply of organic (e.g., amino acids) and inorganic forms (ammonium N [NH_4^+] and nitrate N [NO_3^-]) of nitrogen to plants (Herridge et al., 2008; Dungait et al., 2012). Inhibition of these decomposition

processes by antibiotics could therefore affect access of crop plants to nitrogen, an element essential to efficient plant growth. Effects of antibiotics on nitrogen-fixing plant symbionts, such as *Rhizobia*, which supply up to 80% of total nitrogen in pasture legume plants (Xie et al., 2012), are also possible (Kleineidam et al., 2010; Cevheri, 2012). Thus, antibiotic contamination of agricultural soils could have serious implications for pasture systems.

Despite these plausible mechanisms, evidence demonstrating the effects of antibiotics on plant growth is currently limited. Several laboratory studies indicate that supplementation of growing plants with antibiotics, including metronidazole, oxytetracycline, quinolones, and sulfonamides, can adversely affect the growth and root development of a small number of crop species, specifically alfalfa (Kong et al., 2007), barley (Ferro et al., 2010), soybeans (Jjemba, 2002), and spinach (Aristilde et al., 2010). However, the concentrations used in these experiments were substantially higher than the expected concentrations in agricultural soil and irrigation water. Field studies to confirm these effects and to investigate the influence of environmental parameters, such as temperature and rainfall, are currently lacking. Additional research is needed to determine the extent to which environmental contamination of agroecosystems with active antibiotic compounds poses a threat to plant agriculture.

Less research is available to characterize the impacts of exposure to antibiotics in environmental media on animal agricultural production. Drinking water for agricultural animals typically comes from untreated surface water or groundwater, and there is the potential for these sources to be contaminated with antibiotics at levels greater than those observed in finished domestic drinking water. In addition to drinking water, uptake of antibiotics in livestock feed crops has also been shown (Kumar et al., 2005; Chitescu et al., 2013). Pasture and forage fertilized with agricultural wastewater containing antibiotic residues may also serve as an unintended pathway for exposure. The effects of this potential exposure on animal agriculture, particularly in antibiotic-free settings, are not known and warrant further research.

Potential Effects of Environmental Resistance (Arrows 9–11)

Effects on Human Health

The potential effect of environmental resistance on human health (Fig. 1, arrow 9) has generated a great deal of discussion in recent years (Finley et al., 2013; Wellington et al., 2013; Fernandes et al., 2015), with treatment failures in those treated with antibiotics being the issue of paramount concern to clinicians, administrators, and researchers. Multiple paths by which increased levels of environmental ARB could result in treatment failures have been hypothesized, all of which are thought to occur either through human infection with a pathogenic ARB or uptake of a nonpathogenic ARB and subsequent transfer of resistance genes to pathogens within the human body (Ashbolt et al., 2013). Unfortunately, there is little evidence on which to base quantitative estimates of the effect of environmental resistance on the health of the general public in real-world settings. To our knowledge, epidemiologic studies to assess the relationship of ARB or ARGs in environmental media to human morbidity or mortality have not been done. One study in different regions of India detected no difference in frequency of quinolone resistance (*qnr*) genes in human fecal samples despite a higher prevalence of environmental samples positive for *qnr* genes in one region (Rutgersson et al., 2014). The authors suggested that a high background prevalence of quinolone resistance in human inhabitants of these communities may have masked a true effect of the environmental ARG on human fecal *qnr* content. This is a plausible scenario, and it underscores the importance of undertaking robust epidemiologic investigations of this issue in diverse settings. Using the findings of this study to infer the presence of a human health effect, or lack thereof, is difficult because prevalence of fecal ARGs does not have a known relationship to a human health outcome. Future epidemiologic studies would benefit from the incorporation of an accompanying human health measure or a measure with a known relationship to human health.

Given the paucity of epidemiologic research on the relationship between environmental resistance and human health outcomes, a mathematical modeling approach, known as "human health risk assessment," has been proposed to predict the expected increase in morbidity and mortality due to environmental resistance (Ashbolt et al., 2013). Such an approach requires knowledge or assumptions about, at the least, (i) the amount of ARB in the environmental media, (ii) the rate and pattern of effective human contact with ARB in these media, and (iii) the magnitude of health effect to be expected given such contact (also called the dose–response relationship). Evidence indicates that infections with resistant pathogens are associated with higher costs of treatment, longer hospital stays, and greater risk of death than infections due to susceptible versions of the same pathogens, predominantly due to treatment failures (Centers for Disease Control and Prevention, 2013). A systematic review of transmission of antibiotic resistance from the environment to humans found that, although numerous datasets document the existence of clinically relevant ARB in environmental compartments (e.g., water, soil, and shellfish), no study provided direct evidence for transmission of these pathogens from the environment to humans (Huijbers et al., 2015). Recent work has only just begun to produce quantitative estimates of human exposure to environmental ARB (Leonard et al., 2015). This remains the most significant data gap for overall estimation of human health effects from ARB transmitted via the environment and is likely to hamper any quantitative estimates of risk.

Exposure to the environment, and particularly water use, can be associated with human gastrointestinal illness. Recreational and household use of surface water is a known risk factor for gastrointestinal illness in situations of water-borne outbreaks and under certain environmental conditions (Pruss, 1998; Hamner et al., 2006; Harder-Lauridsen et al., 2013; Yau et al., 2014). Household use of untreated groundwater is also a documented risk factor for sporadic and outbreak-associated gastrointestinal illness (Uhlmann et al., 2009). However, a systematic review of groundwater contamination by enteric pathogens in North America found that exposure assessment for attributing disease to groundwater is a primary gap in knowledge in assessing the influence of groundwater contamination on human health (Hynds et al., 2014). Taken together, this suggests that, although exposure of the general public to bacteria, including ARB, in the

environment is clearly possible, the frequency and patterns of its occurrence are not well understood for most bacterial species.

Specific human subpopulations, such as farm workers, that have prolonged contact with environmental media, surfaces, or animals in agroecosystems may logically be expected to have a higher risk of exposure to ARB in these systems. Although there has been considerable interest in the occupational risk for agricultural workers due to methicillin-resistant *Staphylococcus aureus* (MRSA), a quantitative microbial risk assessment using a conservative approach estimated that the health risk to swine workers was low at one additional MRSA infection among all US farm workers annually (Cox and Popken, 2014). Some studies have found the same or similar antibiotic-resistant strains of *Escherichia coli* and *Enterococcus* spp. in animals, farm environmental samples, and farm workers (Acar et al., 2000; Zhang et al., 2009; Thorsteinsdottir et al., 2010; Deng et al., 2011). This suggests that bacterial transmission between these can occur, although directionality remains unclear. However, to our knowledge, the health effects for agricultural workers from transmission have not been quantified by empirical or modeling approaches. One study found dissimilarity between antibiotic susceptibility of *Clostridium dificile* isolates from swine and associated swine workers, suggesting that transmission from animals to workers or vice versa was unlikely in that cohort (Norman et al., 2014). Chronic occupational exposure to antibiotics in these agroecosystems followed by selection of resident microbiota as discussed above could also contribute to disease risk. Overall, the quantification of health risks for agricultural workers from ARB in agroecosystems other than MRSA has received little research attention.

We also note the importance of considering "low probability but high impact" events, as discussed by Ashbolt et al. (2013). For example, the acquisition by a human pathogen of the New Delhi metallo-β-lactamase-1 gene has had dramatic impacts on treatment efficacy in human clinical medicine in certain settings. This issue has an important place in judging the overall risks posed by anthropogenically influenced resistance in the environment. Standard epidemiologic and quantitative risk assessment methods, which are best used to quantify the properties of time-invariant or recurrent events, are not well suited to address risks associated with events that are extremely rare at the population level. As discussed earlier in this review, the research community can and should work to elucidate the conditions that increase the likelihood of HGT in the environment, especially where human pathogens may acquire ARGs. Mechanistic mathematical modeling approaches, combined with lab- and field-based experimental studies, will be useful in this endeavor.

Effects on Ecosystem Function

We often concern ourselves with the role of natural ecosystems as reservoirs for antibiotic resistance, but science has rarely considered the possible impact of increased levels of ARB or ARGs on these ecosystems themselves (Fig. 1, arrow 10). Although it is plausible that added environmental resistance could cause changes in structure, evolution, and function of microbial communities in natural ecosystems (Martinez, 2009), this relationship remains largely uninvestigated. In one study, bacterial community composition in an aquatic environment was found to differ depending on the quantity of macrolide ARGs, even after adjustment for potential confounding effects of a number of spatial and environmental factors, including antibiotics (Huerta et al., 2013). Despite these efforts to control confounding, the cross-sectional nature of the experimental design hampered the study's ability to fully exclude the possibility that historical levels of environmental antibiotics were the true cause of the differences in microbial populations. To conclusively determine whether ARB affect ecosystems in the absence of environmental antibiotics, experimental trials and longitudinal observational studies will be required. Perhaps most important, whether observed shifts in microbial community composition will result in measurable changes in important ecosystem characteristics, such as nutrient cycling or biodiversity, is not known.

In free-living wildlife, the occurrence of ARB is well documented (e.g., Allen et al., 2010; Greig et al., 2015; Janecko et al., 2015; Smaldone et al., 2014), but we know little about the effect of this resistance on wildlife health. Some have proposed that ingested ARB could affect the intestinal microbiomes of wildlife species, leading to changes in the functional properties of these bacterial communities and perhaps to health consequences for the animal (Gillings, 2015). However, this hypothesis has not been investigated. It may be more likely that ingestion of environmental antibiotics by wildlife would result in such an effect, as discussed above in the section on environmental antibiotics and ecosystem function.

Effects on Agricultural Systems

Bacterial pathogens present in agroecosystems, especially ARB, are likely to have an impact on the efficiency with which agricultural systems are able to humanely produce high-quality, healthful food products for human consumption (Fig. 1, arrow 11). There is evidence that agroecosystems, in general, and water, in particular, are an important source of bacterial transmission to food animals (Kinde et al., 1996) and contamination of crops (Berger et al., 2010; Erickson et al., 2014; Martinez et al., 2015), although few studies have sought to document an association between environmental ARB, specifically, and ARB in either agricultural animals or crop plants (Greig et al., 2015). A scoping review of the role of wildlife in transmitting ARB to the food chain found a large body of evidence documenting an association between wildlife, particularly wild birds, and common foodborne pathogens, including ARB, in agricultural products (Greig et al., 2015). Although such associational studies are not necessarily evidence of a directional transfer of ARB from environmental sources to agricultural systems, such transfer is indeed likely because animals and crops live intimately with their environment regardless of whether they are raised under field or confinement conditions. For example, most animals in agriculture globally consume water that is directly taken from surface or groundwater sources with minimal treatment. Similarly, crops are irrigated with untreated ground and surface water or reclaimed wastewater. As described above, ARB and ARGs are commonly detected in these water sources, thus potentially exposing agricultural systems to this resistance.

Advances in laboratory and analytic methods, particularly whole genome sequencing and phylogenetic analysis, are useful in inferring the direction of bacterial transmission. Studies using genomic epidemiology have been used to improve understanding of bacterial population connectivity for *Salmonella* in Scotland

(Mather et al., 2012; Mather et al., 2013) and tuberculosis among human contact networks in western Canada (Gardy et al., 2011; Wlodarska et al., 2015). Such methods have not yet been applied to transmission of bacteria from environmental sources to agriculture (Greig et al., 2015). Their increasing usage will be useful in understanding the flow of ARB in agroecosystems and are discussed further in an accompanying review (Luby et al., 2016).

Key Knowledge Gaps and Research Considerations

There remain important gaps in our understanding of the role that antibiotics and antibiotic resistance in agroecosystems play for human health, ecosystem function, and agricultural system productivity (Fig. 4). In particular, the causal model presented in Fig. 1 highlights a broad knowledge gap with respect to the effects of environmental antibiotics, ARB, and ARGs. Although plausible mechanisms for these effects exist (Fig. 1, arrows 6–11), little data are available to assess the magnitude of those effects.

Despite the fact that research has primarily been driven by the potential human health effects of ARB in the environment (Fig. 1, arrow 9), those effects remain difficult to quantify, and the dearth of exposure data prevents meaningful quantitative risk assessment. Evidence strongly suggests that transmission of bacteria, including ARB, from environmental sources to humans and agricultural systems can occur, but more work is needed regarding ARB specifically. The frequency and pattern of bacterial transmission events from the environment to humans, including agricultural workers, as well as to food animals and crops is as yet unclear for most ARB. Thus, the effects of ARB in agroecosystems, specifically, and the environment, more generally, on human health (Fig. 1, arrow 9) and agricultural systems (Fig. 1, arrow 11) also remains unclear at the population level.

Similarly, the effects on natural ecosystems from antibiotics and antibiotic resistance in agroecosystems have received very little research attention (Fig. 1, arrows 7 and 10). Overall, improved risk characterization will require a better understanding of exposure levels in locations across the world and empirical investigations documenting the associated effects in humans, natural ecosystems, and agricultural systems. As work progresses in this research area and in others reviewed here, proxy outcomes, such as fecal ARGs or microbial community composition, that do not have an established relationship to outcomes of interest should be used cautiously and coupled with studies to improve understanding of their meaning.

Unknowns involved in the effect of antibiotic use on antibiotics, ARB and ARGs in environmental compartments, generally, and agroecosystems, in particular, are also important research gaps because this region of the causal system (Fig. 1, arrows 1–5) is well suited for intervention. To develop effective interventions around agricultural antibiotic use and waste handling practices, research is needed to address several gaps in knowledge.

For example, a detailed mechanistic understanding of the fate and transport of antibiotics in agroecosystems (Fig. 1, arrow 1) is lacking for certain medically important antibiotic classes, especially aminoglycosides and β-lactams, making it difficult to develop accurate landscape-scale models for the prediction of human antibiotic exposure via the environment. Poor characterization of mixture effects and the fate, transport, and availability of sediment-associated antibiotics to microbial populations also complicates efforts to predict the concentration of active antibiotics in environmental systems.

Key knowledge gaps

- Level of exposure of humans and agricultural systems to ARB and ARGs in the environment and dose-response effects of these exposures (Fig. 1, arrows 9 and 11)
- Effect of antibiotics and ARB on wildlife and natural ecosystems (Fig. 1, arrows 7 and 10)
- Mechanistic understanding of fate and transport for aminoglycosides and β-lactams (Fig. 1, arrow 1)
- Relative impact of released antibiotics and selection for resistance (Fig. 1, arrows 1 and 4) versus released ARB/ARGs on resistance in the environment (Fig. 1, arrow 2)
- Contribution of horizontal gene transfer (HGT) to resistance in the environment (Fig. 1, arrow 5)

Fig. 4. List of key knowledge gaps regarding antibiotics and antibiotic resistance in agroecosystems.

Another example is the lack of knowledge about the extent to which antibiotic use increases the prevalence of antibiotic resistance in agroecosystems directly (Fig. 1, arrow 2) or indirectly through antibiotic-induced selection in the environment (Fig. 1, arrows 1 and 4). The relative contribution of these two causal paths remains unclear and is an important knowledge gap. Closing this gap will permit a better understanding of the amount and type of ARB expected in anthropogenically affected environmental systems and, subsequently, improve intervention efforts. Research on the influence of and the factors affecting HGT in environmental bacterial communities (Fig. 1, arrow 5) is also urgently needed to improve risk assessments of this issue and to facilitate interpretation of the large volume of research underway on environmental ARGs. The extent to which certain environmental conditions may foster increased rates of HGT is an issue worthy of attention.

Causal models similar to Fig. 1 may offer a valuable tool to researchers in this area, particularly where experimental control of all relevant factors is not possible and observational research is a necessity (Singer and Williams-Nguyen, 2014). Such a model can be of tremendous value in identifying factors that may confound or mediate the relationship of interest (Shrier and Platt, 2008) in a particular study. For example, where a researcher is interested in the relationship between ARB and ecosystem function (Fig. 1, arrow 10), examination of Fig. 1 shows that antibiotics in the environment should be considered as a possible confounding variable. This is because increased concentrations of antibiotics in the environment may cause both an increased concentration of ARB in that environment (Fig. 1, arrow 4) and effects on ecosystem function (Fig. 1, arrow 7). Thus, environmental occurrence of antibiotics may serve as an alternate explanation for an observed association. In such a scenario, study design or analysis methods can help to control this potential confounding effect. Although confounders are already recognized and addressed in many studies in the field, this is not universally true. In particular, we suggest that possible confounding by current and historical levels of environmental antibiotics should be

considered in studies estimating the effect of ARB in the environment on human, ecosystem, or agricultural outcomes (Fig. 1, arrows 9–11).

Conclusions

We propose a simple causal model (Fig. 1) that describes relationships between antibiotics, ARB, ARGs, and three critical endpoints to serve as a guiding structure for understanding what is currently known about antibiotic resistance in agroecosystems. Interest in this topic has typically focused on possible risks to human health. Here we take a One Health approach, wherein we consider possible effects on humans, natural ecosystems, and the agricultural system itself, recognizing that these are all interrelated in the long term. This approach is supported by a recent comprehensive action plan to address antibiotic resistance released in 2015 by the US executive branch, which emphasizes the importance of a "One Health" approach for combating antibiotic-resistant bacteria (President's Council of Advisors on Science and Technology, 2015).

We have presented a review of the current state of the science on antibiotics and antibiotic resistance in agroecosystems structured around this simple causal model and have identified overarching gaps in knowledge for which information is urgently needed. An improved understanding of these causal relationships in specific systems will permit better targeting of mitigation strategies to reduce occurrence of environmental resistance and its potential associated effects. We put forward these research priorities and urge researchers and regulators to embrace them so that we may move the state of the science forward.

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